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Control of Cilia in the Branchial Basket of *Ciona intestinalis* (Ascidacea)

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Abstract. We investigated arrest and inactivation responses of stigmatal cilia in the branchial basket of the ascidian, *Ciona intestinalis*. Using an improved preparation of living tissue for microscopic imaging of ciliary responses, we found that Ca-ionophore A23187 in seawater + 50 m*M* Ca caused actively beating cilia to assume the upright inactive posture, while A23187 in seawater + 100 m*M* Ca caused transient (5–10 s) stigma-wide arrests in which the cilia lie flat against the stigmatal walls. Both responses are therefore Ca dependent, but the inactive state has a lower threshold for Ca than does arrest. Membrane permeant cAMP analogues induced >40% of the quiescent cilia within a stigma to begin beating.

Saponin-extracted models of stigmatal cilia were developed to study the ionic and molecular control of ciliary activity in *Ciona*. Extracted cilia were stimulated to beat vigorously for >45 min by ATP-containing reactivation solution (RS). Addition of 10^{-5} to 10^{-3} *M* Ca to reactivation solution caused the cilia to stand upright (inactivate), but not to arrest. The calmodulin antagonists trifluoperazine and calmidazolium (100 μM) restored active beating when included in RS + 50–100 μM Ca, thereby reversing Ca-dependent inactivation. Addition of bovine brain calmodulin to RS + 100 μM Ca did not cause arrest of reactivated cilia. RS + 100 μM cAMP + 1 m*M* 3-isobutyl-1-methyl-xanthine or the catalytic subunit of c-AMP-dependent protein kinase increased both the proportion and vigor of reactivated beating. Addition of 100 μM Ca to the RS + cAMP + IBMX solution caused reactivated cilia to vibrate or twitch in an upright position, suggesting that Ca and cAMP have antagonistic effects on stigmatal cilia.

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Abbreviations: B-cAMP, N6-benzoyl-cAMP; M-cAMP, N6-monobutryl-cAMP; ES, extraction solution; WS, wash solution; RS, reactivation solution; TFP, trifluoperazine; IBMX, 3-isobutyl-1-methyl-xanthine; TAME, Nα-p-tosyl-L-arginine methyl ester, PKA, c-AMP-dependent protein kinase.

Introduction

The activity of cilia and flagella is regulated in many organisms, reflecting the important role of these organelles in locomotion, suspension feeding, gas exchange, mucous and gamete transport, and sperm chemotaxis. Well-known examples of ciliary and flagellar responses to stimuli include reorientation of beat direction in ciliate protozoa (Eckert *et al.*, 1976; Machemer, 1986), arrest of lateral cilia of lamellibranch gills (Murakami and Takahashi, 1975; Tsuchiya, 1977; Walter and Satir, 1978), inhibition of velar cilia of molluscan larvae (Carter, 1926), activation of *Mytilus* abfrontal cilia (Stommel, 1984), *Beroë* macrocilia (Tamm, 1988), and sperm flagella (Brokaw, 1987), changes in waveform of sea urchin sperm flagella (Brokaw *et al.*, 1974; Brokaw, 1979) and *Chlamydomonas* flagella (Hyams and Borisy, 1978; Bessen *et al.*, 1980), and reversal of direction of wave propagation in trypanosome flagella (Holwill and McGregor, 1976). Many of these axonemal responses are known to be triggered by depolarization-induced changes in intracellular Ca or alterations in cyclic nucleotide levels (Machemer, 1986; Brokaw, 1987; Otter, 1989; Stephens and Stommel, 1989; Preston and Saimi, 1990; Bonini *et al.*, 1991).

Tunicates show periodic interruptions in the beating of cilia that line openings (stigmata) of the branchial basket and generate the feeding current (Fedele, 1923; MacGinitie, 1939). These temporary ciliary arrests occur spontaneously, in response to general disturbances, or when undesirable material enters the branchial siphon (Bone

and Mackie, 1982), and are usually accompanied by quick contractions of siphons and mantle ("squirts") (MacGinitie, 1939). Previous studies on the ascidian branchial basket showed that ciliary arrests are: (1) induced by depolarizing stimuli, (2) controlled by identified neurons in the central ganglion, (3) mediated by cholinergic synapses, (4) correlated with action potentials recorded from the stigmatal ciliated cells, and (5) dependent on external Ca (Takahashi *et al.*, 1973; Mackie *et al.*, 1974; Arkett, 1987; Arkett *et al.*, 1989). Ascidian stigmatal cilia also undergo an "inactive" state in which they stand in an upright position before resuming normal beating (Takahashi *et al.*, 1973; Mackie *et al.*, 1974; Arkett, 1987).

Despite the evidence suggesting that ascidian cilia are controlled by mechanisms similar to those operating in other systems, neither Ca dependence of arrest or inactivation responses, nor the possible role of cyclic nucleotides in ciliary activity has been investigated directly in any tunicate.

We have addressed these questions in the ascidian *Ciona intestinalis* by devising an improved method for microscopic imaging of motile responses of living cilia, and by developing the first detergent-extracted, ATP-reactivated cell models of stigmatal cilia. These new advances enable direct tests of the ionic and biochemical basis of control of ciliary motility in *Ciona* and are valuable complements to other studies on regulation of sperm flagellar motility in *Ciona* (Brokaw, 1987). A preliminary report of this work has appeared (Bergles and Tamm, 1989).

Materials and Methods

Organism

Specimens of *Ciona intestinalis* (5–10 cm long) were obtained from Marine Resources at the Marine Biological Laboratory and kept in baskets immersed in running seawater, or were simply removed from the sides of laboratory sea-tables in which they had settled and grown. *Ciona* specimens were used within a week after removal from their substrate, because the branchial basket often deteriorated after the animals were detached.

Perfusion slides of living stigmatal cilia

Ciona were pinned laterally against a Sylgard-coated dish, and the tunic was sliced open and pulled back. The branchial cavity was opened by cutting longitudinally along the length of the endostyle, then across between the two siphons. The branchial basket was removed by cutting the many trabeculae connecting the basket to the mantle. The excised branchial basket was pinned flat in a Sylgard-lined petri dish of normal seawater and kept on ice at 0°C. Prior to experimentation, the branchial basket was further subdivided into small rectangular pieces, about 1 × 5 mm, by cutting between the longitudinal bars with fine iridectomy scissors. Tissue was used within 2–4 h after its removal from the animal.

In the final preparation, a piece of branchial basket was pipetted onto a microscope slide that had been ringed by a 15 × 50 mm rectangular ridge of petroleum jelly (Vaseline). The tissue was stretched out near the center of this rectangular well with the pharyngeal side facing upward. The ends of the piece were then pressed down against the slide by fine stainless steel pins anchored in dabs of Vaseline that had previously been placed on the dry slide. A square coverslip was placed over the tissue, mounted near the center of the rectangular well, leaving room on either side of the coverslip to add and withdraw solution from the well during perfusion.

Cell models

Small rectangular pieces of branchial basket were placed in 0.1% saponin, 1% DMSO, 20 m*M* EGTA, 150 m*M* KCl, 10 m*M* $MgCl_2$, 30 m*M* PIPES, pH 7 (extraction solution, ES) in a glass well for 4–9 min at room temperature. Tissue was transferred to a second well containing 2 m*M* ATP, 1 m*M* DTT, 10 m*M* EGTA, 150 m*M* KCl, 10 m*M* $MgCl_2$, 30 m*M* PIPES, pH 7 (reactivation solution, RS), or to the same solution without ATP (wash solution, WS). The tissue pieces were then mounted on perfusion slides for observations. Extraction and reactivation of living pieces mounted on perfusion slides was not feasible due to distortion of the tissue by muscular contractions induced by ES.

The effects of ions or reagents on ciliary reactivation were estimated as follows (Table I). Twenty-five to 100 stigmata with clearly observable cilia were observed for each tissue piece. A rating of ++++ indicates that >95% of the cilia in each stigma were beating vigorously. Ratings of +++, ++, and + indicate that 75–95%, 50–75%, and 15–50% of the cilia in each stigma were beating, respectively, typically at decreasing frequencies. A rating of ± indicates that 5–15% of the cilia were active, and a negative rating indicates that <5% were beating. All treatments were repeated on at least five different preparations of branchial basket pieces.

Reagents and solutions

Calmidazolium and norepinephrine were obtained from Calbiochem-Behring Corp. (San Diego, California); sodium metavanadate was obtained from Mallinckrodt Inc. (St. Louis, Missouri). Calmodulin (bovine brain), cAMP (bovine brain), protein kinase catalytic subunit (bovine heart), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri). Ca-EGTA

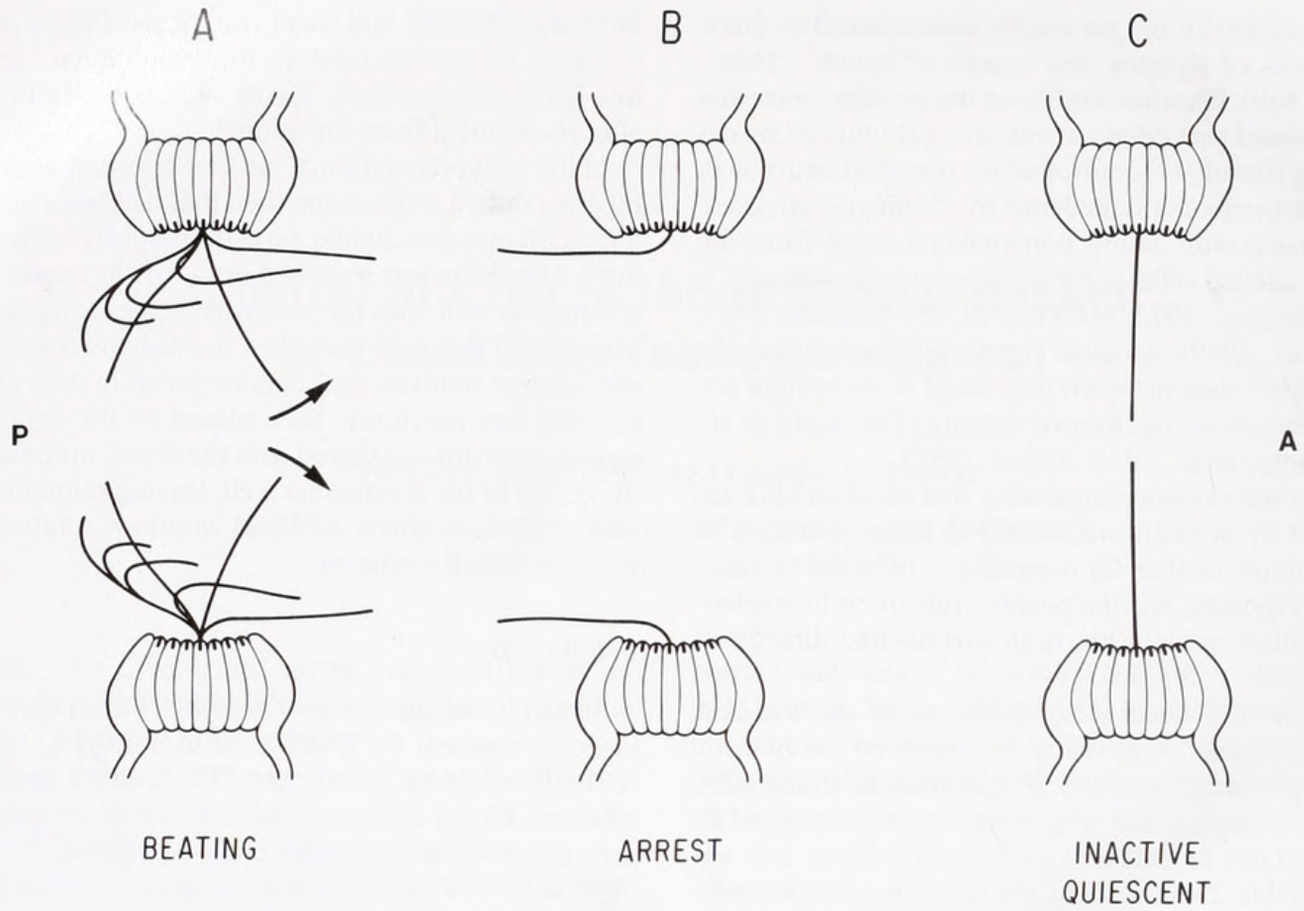

Figure 1A–C. Diagram of three different states of *Ciona* stigmatal cilia, as viewed in cross section of a stigma to show ciliary profiles. For clarity, only one of the cilia arising from the seven ciliated cells is shown. The branchial cavity (P) is to the left, the atrial (cloacal) cavity (A) is to the right in all figures. Based on our observations and Takahashi *et al.* (1973), Mackie *et al.* (1974), and Arkett (1987). (A) Active cilia beat with the effective stroke directed towards the atrial chamber (arrows), propelling water out of the pharynx. The recovery stroke occurs in three dimensions, out of the plane of the power stroke. Metachronal waves (not shown) travel at right angles to the effective stroke. (B) Arrested cilia lie flat against the stigmatal walls, inclined in a posture beyond the end of the recovery stroke (stigmata open). (C) Following arrest, cilia stand upright in an inactive posture (stigma closed) before active beating resumes. Quiescent cilia also remain in this position.

buffers were prepared according to Salmon and Segall (1980).

Light microscopy

Perfusion slides were viewed with Zeiss brightfield or phase-contrast optics (16×/0.40 NA or 40×/0.75 NA objectives), and images were recorded with a DAGE 67M video camera (Dage-MTI, Michigan City, Indiana 46360) on a VHS videocasette recorder allowing still-field playback (GYYR model 2051, Anaheim, California 92802). Beat frequency was determined by repetitive counting of the number of video fields (1/60 s) per beat cycle. Photographs of still-fields from a video monitor were taken with an Olympus OM-2N camera on Kodak Tech Pan (2415) 35 mm film.

Results

Stigmatal ciliary system

The anatomy and motility of the stigmatal ciliary system of *Ciona* and other ascidians have been described previously (MacGinitie, 1939; Takahashi *et al.*, 1973; Mackie *et al.*, 1974; Arkett, 1987), and are reviewed briefly here.

The branchial slits or stigmata are lined with seven rows of laterally flattened cells, each bearing a single row of cilia (Fig. 1). The ciliated cells are stacked side-by-side in clusters that abut end-to-end, forming a continuous ciliated band around the inside of a stigma. Neurons run within the blood sinus and make synaptic contacts onto the bases of the ciliated cells, which are coupled by gap junctions (Mackie *et al.*, 1974; Arkett *et al.*, 1989).

The stigmatal cilia beat outward from the branchial cavity towards the atrial cavity, generating a water current that enters the branchial (incurrent) siphon, passes through the branchial basket, and flows out the atrial siphon (Fig. 1A). Ciliary beating is coordinated into dexioplectic metachronal waves that travel unidirectionally around the stigmatal openings (Fig. 2A).

In response to mechanical, electrical, or chemical stimulation, all the cilia lining a stigma perform a single rapid reverse stroke and lie flat against the stigmatal walls for 1–2 s in an arrest position inclined beyond the end of the normal recovery stroke (Fig. 1B). Ciliary arrest halts water flow into the pharynx and leaves the stigmata completely open, allowing muscular contractions to "squirt" water out of the branchial siphon.

Following an arrest, the cilia gradually rise to an upright position, closing the stigmatal opening (Fig. 1C). The cilia remain in this straight "inactive" state for a few seconds before beating resumes and normal metachrony is re-established.

In both excised pieces of branchial basket and exposed intact baskets, some stigmata are always observed with cilia that stand upright in an "inactive" posture for long periods. It is not known whether this long-lasting inactive state was, in any case, preceded by an arrest; but a direct transition from beating to the inactive position has never been reported (Takahashi *et al.*, 1973; Mackie *et al.*, 1974; Arkett, 1987). Upon stimulation, inactive cilia as well as beating cilia perform an arrest response together (Takahashi *et al.*, 1973; Mackie *et al.*, 1974).

Effects of calcium ionophore

Perfusion of pieces of branchial basket with 100 μM A23187 in normal seawater for 15–30 min had no noticeable effect on stigmatal ciliary activity. Addition of 50 mM $CaCl_2$ to both the bath and the ionophore suspension caused most of the cilia to assume an upright inactive position (stigmata closed) within 5 s after perfusion of ionophore. Cilia remained in this posture for as long as observed (up to 5 min). The addition of 100 mM $CaCl_2$ to the bath and ionophore suspension resulted in stigma-wide ciliary arrests throughout the field of view within 5 s after perfusion of A23187 (Fig. 2). Ciliary arrests lasted less than 5–10 s, after which the cilia moved to the inactive position and remained upright for 15–30 s before resuming beating. When larger pieces of branchial basket were used, arrests were accompanied by vigorous muscular contractions.

Perfusion of normal seawater containing 50 mM or 100 mM Ca without A23187 but with solvent (0.1% ethanol/DMSO) did not elicit arrests or inactivations.

Membrane-permeant cAMP analogues

To investigate the possible role of cAMP-regulated processes (*i.e.*, activation of PKA) in stigmatal ciliary responses, we applied membrane-permeant cAMP analogues to branchial basket pieces on perfusion slides.

We directed our attention to stigmata where most of the cilia were standing upright in a long-lasting inactive state. Perfusion of 1–10 mM N6-benzoyl-cAMP (B-cAMP) or N6-monobutyryl-cAMP (M-cAMP) in normal seawater caused many of the quiescent cilia to beat with normal metachronal coordination within 1–3 min. In most cases, more than 40% of the cilia became active. A greater number of quiescent cilia became active in stigmata where some of the cilia were already beating prior to perfusion with B-cAMP or M-cAMP. No significant activation of cilia was observed after perfusion of the solvent carrier (0.1% ethanol) in seawater without cAMP analogues.

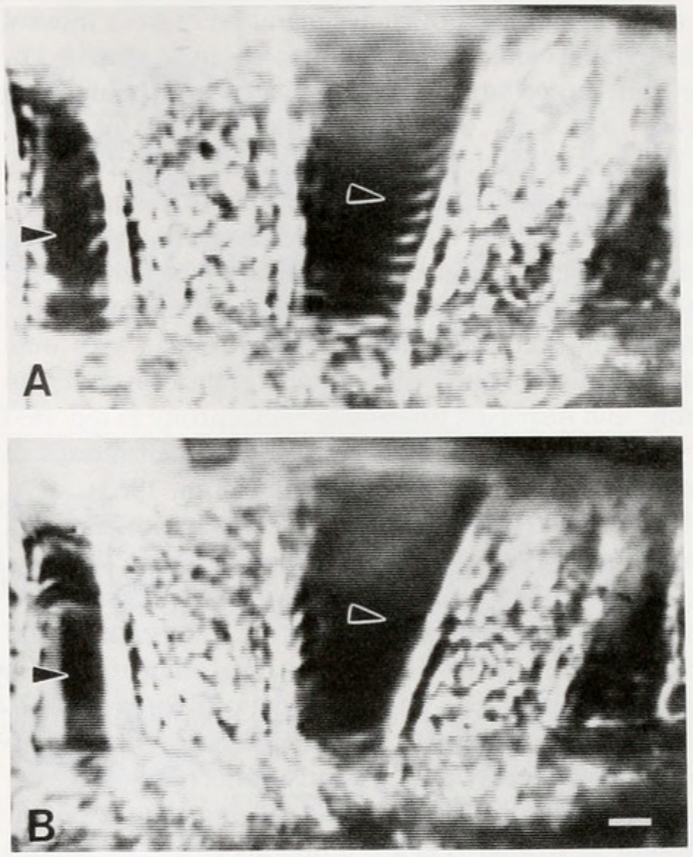

Figure 2. Ca ionophore-induced arrest of stigmatal cilia. (A) In seawater, metachronal waves of ciliary activity are evident on the stigmatal wall (arrowhead). (B) Perfusion of A23187 in seawater + 100 mM Ca causes ciliary arrest (arrowhead). Video prints; scale bar, 20 μm.

Cell models

Extraction and reactivation. Treatment of pieces of branchial basket in ES for 4–9 min stopped most stigmatal cilia in a more or less upright position. The cilia projected as tufts from clusters of swollen stigmatal cells, and there were gaps between the ciliary tufts of adjacent cell groups. Transfer of tissue to WS did not activate beating; the cilia remained in a relatively upright posture (Fig. 3A). Shorter extraction times (2–3 min) resulted in very slow ciliary beating (<2 Hz) in WS; the cilia usually stopped in the inactive position within 5–10 min. Thin-section electron microscopy of tissue extracted for 7 min showed partial

or complete removal of ciliary membranes from most of the stigmatal cilia, while non-extracted tissue prepared by the same procedure had intact ciliary membranes (not shown).

Transfer of extracted branchial basket tissue to RS resulted in vigorous beating of 50–70% of the stigmatal cilia. Ciliary reactivation typically lasted more than 45 min. Beating sometimes dislodged or displaced the ciliated cells from the fragile stigmatal wall, causing them to "swim" through the solution. Normal metachronal waves were not present. Separated tufts of cilia often beat independently, and displayed unicellular metachrony as reported for reactivated lateral cilia on separated cell groups of *Modiolus demissus* gills (Child and Tamm, 1963).

Reactivated beating was also observed by perfusing RS through a slide of extracted branchial basket in WS (Fig. 3B). RS perfusion caused the cilia to beat hesitantly at first with a restricted range of motion, then rapidly and fully within 10–20 s. Reactivated cilia often reached a steady-state frequency similar to that of living cells (10–14 Hz). Long stretches of cilia sometimes beat nearly synchronously to form common wavefronts (Fig. 3B).

Vanadate inhibition. RS + 20 μM vanadate, a potent inhibitor of dynein ATPase (Gibbons *et al.*, 1978), did not reactivate ciliary beating. Norepinephrine (5 mM) in RS + vanadate restored reactivated beating, reversing vanadate inhibition of motility (Table I).

Ca sensitivity. ES-treated pieces of branchial basket showed normal reactivation of ciliary beating when placed in wells of 10^{-7} to 10^{-6} M free Ca (Ca-EGTA buffer). However, RS + 10^{-5} to 10^{-3} M Ca caused the majority of cilia to assume an upright inactive posture, but never an arrest position (Table I). A gradual decrease in Ca sensitivity for eliciting inactivation was observed with longer times in ES; extraction times of more than 9 min often yielded cilia that did not inactivate in response to Ca.

To check whether a transient Ca-induced arrest response might have been missed in depression wells, RS + 10^{-4} to 10^{-3} M Ca was perfused into a slide containing a piece of extracted branchial basket in WS. No momentary arrest response was observed before the cilia assumed a rigidly straight inactive position.

Various approaches were tried to elicit arrests in RS + 10^{-5} to 10^{-3} M Ca. For example, a cocktail of protease inhibitors (1 mg/ml trypsin inhibitor; 0.5 mg/ml leupeptin; 1 mM TAME; 0.2 mg/ml PMSF, 1 mg/ml BSA) were included in both ES and RS to prevent possible proteolysis of putative Ca sensors or proteins required to mediate arrest. Different detergents (Brij-58, Brij-35, Triton-X 100) were tried in place of saponin to preclude the extraction of Ca-binding proteins (*i.e.*, calmodulin). In another series of experiments, K acetate was substituted for KCl in both ES and RS. None of these modifications resulted in ciliary arrests in RS + 10^{-4} M Ca. We did note, however, that reactivation was consistently better in solutions containing KCl rather than K acetate (Table I).

Calmodulin. Addition of calmodulin antagonists, 100 μM trifluoperazine (TFP) or 100 μM calmidazolium to RS + 50–100 μM Ca restored reactivated beating, thereby reversing Ca-dependent inactivation (Table I). TFP typically gave more consistent results than did calmidazolium. Chlorpromazine (100 μM) did not significantly reverse the inactivation of cilia in RS + Ca. These results indicate that calmodulin mediates Ca-dependent inactivation of stigmatal cilia.

Addition of 65 μg/ml bovine brain calmodulin to RS + 100 μM Ca did not elicit an arrest of cilia in tissue initially bathed in WS or RS (Table I).

cAMP and PKA

RS + 100 μM cAMP and 1 mM IBMX, a cyclic nucleotide phosphodiesterase inhibitor substantially increased both the proportion and vigor of ciliary reactivation compared to tissue incubated in RS alone (Table I). Thus cAMP and Ca exert opposing effects on ciliary reactivation. In RS containing 100 μM cAMP + 100 μM Ca, cilia vibrated or twitched rapidly in a rigid inactive position. Addition of 28 μg/ml catalytic subunit of PKA to RS likewise improved the extent of ciliary reactivation to more than 95% in most cases (Table I).

Discussion

Calcium and arrest

The role of Ca in triggering a variety of ciliary and flagellar motor responses is well documented (Eckert and Murakami, 1972; Naitoh and Kaneko, 1972; Tsuchiya, 1977; Hyams and Borisy, 1978; Walter and Satir, 1978; Gibbons and Gibbons, 1980; Brokaw and Nagayama, 1985; Nakamura and Tamm, 1985; Satir, 1985; Stommel and Stephens, 1985; Machemer, 1986; Brokaw, 1987, 1991; Tamm, 1988; Otter, 1989).

Although arrest of stigmatal cilia in ascidians has long been suspected to be Ca-dependent (Takahashi *et al.*, 1973; Mackie *et al.*, 1974), direct evidence for this has been lacking. Our finding that Ca ionophore in the presence of 100 mM Ca elicits arrest of *Ciona* stigmatal cilia strongly argues for the Ca-dependency of this response. However, these experiments were performed on pieces of branchial basket tissue, and stigmatal cilia have been shown to be under neuronal control (Mackie *et al.*, 1974; Arkett, 1987). Therefore, our results could also be explained by ionophore-mediated influx of Ca at presynaptic sites mediating nervous control of ciliary arrest, without requiring Ca influx into the ciliated cells themselves.

To directly test whether ciliary motility in ascidians is regulated by Ca, we prepared the first ATP-reactivated

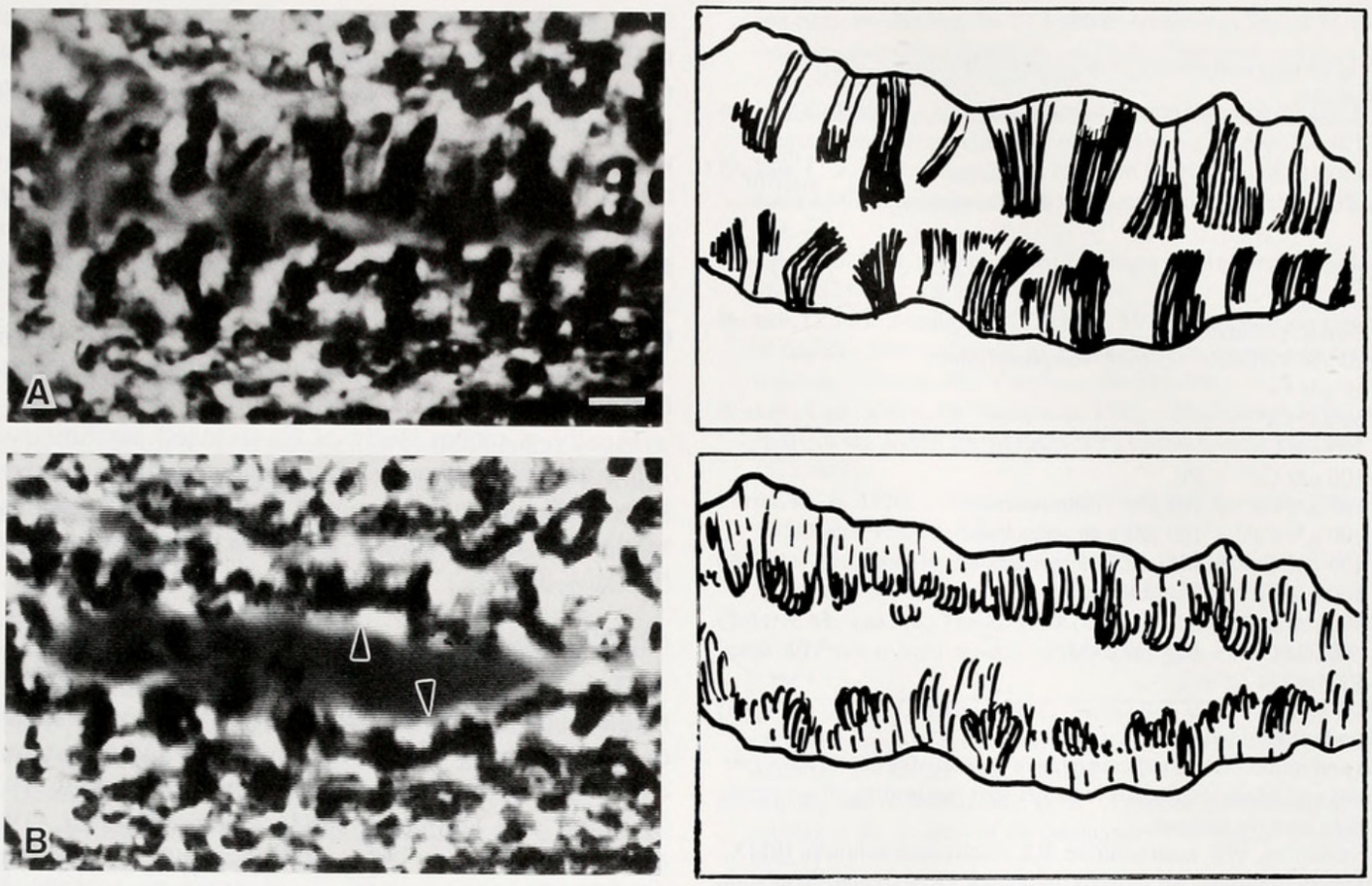

Figure 3. Stigmatal cell models. Cilia lining a stigma are shown on the left and diagrammed on the right. (A) A single stigma in WS. Tufts of immotile cilia (black) stand upright, projecting from the refractile wall of the stigma into the stigmatal space. In the diagram, the wall (edge) of the stigma is indicated by the irregular horizontal lines. (B) The same stigma after perfusion of RS. The cilia beat vigorously and, to a large extent, synchronously, giving rise to common wavefronts (arrowheads). As a result, the stigma is more open than in A. The stigmatal wall does not change (compare outlines of stigma in diagrams). Scale bar, 10 μm, *A, B*.

models of stigmatal ciliated cells. We were unable to elicit ciliary arrest in our saponin-permeabilized models at any Ca concentration used (10^{-5} to 10^{-3} *M* Ca in RS). Instead, the axonemes stopped in an upright inactive position without passing through an arrest.

We were concerned that our permeabilization procedure, or possibly subsequent proteolysis, might have removed or destroyed critical control factors or Ca-binding proteins (*i.e.,* calmodulin) necessary for demonstrating Ca-sensitivity of arrest in cell models. For example, extraction of calmodulin from sea urchin sperm and protist cilia leads to modification or loss of Ca control of axonemal motor responses (Brokaw and Nagayama, 1985; Izumi and Miki-Noumura, 1985; Izumi and Nakaoka, 1987). Extraction and incubation procedures may also modify the calmodulin-binding affinity of the axoneme (Brokaw, 1991). In addition, some detergents commonly used to make cell models (*i.e.,* Triton-X 100) are potent inhibitors of both calmodulin and calmodulin-dependent cyclic nucleotide phosphodiesterase (Sharma and Wang, 1981).

However, our attempts to restore presumed Ca sensitivity of ciliary arrest in models by trying different detergents, extraction times, protease inhibitors, or addition of exogenous bovine brain calmodulin, were uniformly unsuccessful. Nevertheless, the variable sensitivity of the inactivation response of models to Ca, particularly after longer extraction times (more than 9 min in ES), suggests that the absence of Ca-sensitive arrest in our models may be due to loss or modification of an as yet unidentified factor.

Calcium and inactivation

The upright inactive posture of stigmatal cilia is clearly Ca-dependent: reactivated cilia are inactivated by 10^{-5} to 10^{-3} *M* Ca, and cilia on living tissue are inactivated by Ca ionophore in the presence of a lower Ca concentration (50 m*M*) than that leading to arrest (100 m*M*, see above). These findings suggest that inactivation has a lower threshold to intracellular Ca than does arrest.

Inactive cilia of cell models in RS + Ca were induced to beat by the addition of a calmodulin antagonist, either

Table I

Effects of various compounds on reactivation of ciliary motility in cell models

Solution	Ciliary activity
WS	–
RS (KCl)*	++(+)
RS (K Acetate)	++
RS + 20 μM Vanadate	–
RS + 20 μM Vanadate + 5 mM Norepinephrine	+++
RS + 10 μM Ca^{2+}	+
RS + 50 μM Ca^{2+}	±
RS + 100 μM Ca^{2+}	–
RS + 100 μM Ca^{2+} + PIs	–
RS + 100 μM Ca^{2+} + 100 μM Trifluoperazine	+++
RS + 100 μM Ca^{2+} + 100 μM Calmidazolium	++
RS + 100 μM Ca^{2+} + 100 μM Chlorpromazine	±
RS + 100 μM Ca^{2+} + Calmodulin (65 μg/ml)	–
RS + 100 μM cAMP + 1 mM IBMX	++++
RS + 100 μM Ca^{2+} + 100 μM cAMP	– vibrating
RS + PKAcs (28 μg/ml)	++++

* Standard RS used below.

Plus and minus ratings indicate relative degrees of ciliary activity (see Materials and Methods); minus indicates that nonbeating cilia were in an upright inactive position.

Abbreviations: WS, wash solution; RS, reactivation solution; IBMX, isobutylmethylxanthine; PIs, protease inhibitors; PKAcs, catalytic subunit of protein kinase.

TFP or calmidazolium. This suggests that Ca-induced inactivation of *Ciona* stigmatal cilia is mediated by calmodulin. Other Ca-dependent ciliary motor responses, such as arrest of mussel gill lateral cilia (Reed *et al.*, 1982; Stommel, 1984), activation of *Mytilus* gill abfrontal cilia (Stommel, 1984), and reorientation of ciliary beat direction in some cell models of *Paramecium* (Otter *et al.*, 1984; Izumi and Nakaoka, 1987) and *Tetrahymena* (Izumi and Miki-Noumura, 1985), are also partially or completely inhibited by anti-calmodulin drugs.

Ca may exert its effects on ciliary motility by activating calmodulin-dependent protein kinase (C kinase) or phosphatase (calcineurin), thus changing the phosphorylation levels of axonemal regulatory proteins (Nakaoka and Ooi, 1985; Tash, 1989; Hamasaki *et al.*, 1989; Bonini *et al.*, 1991). Because the catalytic subunit of PKA did not inactivate reactivated *Ciona* stigmatal cilia, but rather enhanced motility, the mechanism by which Ca-calmodulin is presumed to inactivate stigmatal cilia may involve a dephosphorylation reaction.

The transient inactive state exhibited by *Ciona* cilia after every arrest resembles the transient inactivation of *Paramecium* cilia that occurs after depolarization-induced Ca-dependent reversal of beat direction, before the beat cycle is renormalized (Machemer, 1986). Voltage-clamp experiments with *Paramecium* showed that an inactive state, or frequency minimum, also intervenes between normal beating and the onset of stimulus-induced ciliary reversal. In *Paramecium*, a transient inactivation response therefore precedes and follows the Ca-dependent ciliary reversal response, suggesting that inactivation may be caused by a Ca concentration slightly elevated above normal resting level (Machemer, 1986). The transient inactive state following the arrest of *Ciona* cilia may also reflect an intermediate level of internal Ca concentration. The epaulette cilia of echinoplutei larvae sometimes undergo a similar upright inactive state after Ca-dependent reversed beating (Mogami *et al.*, 1991).

Finally, a recent study of Ca-induced asymmetry of ATP-reactivated flagellar bending waves of sea urchin sperm indicates the existence of two separate Ca responses, mediated by high-affinity and lower-affinity Ca sensors (Brokaw, 1991).

cAMP and quiescence

Cyclic nucleotides (cAMP, cGMP) also play a role in regulating ciliary and flagellar motility: in particular, cAMP is typically involved in initiating and maintaining ciliary and flagellar beating (Opresko and Brokaw, 1983; Stommel and Stephens, 1985; Takahashi *et al.*, 1985; Murofushi *et al.*, 1986; Brokaw, 1987; Murakami, 1987a,b; Stephens and Stommel, 1989; Tash, 1989; Bonini *et al.*, 1991).

Neuronal activation of quiescent lateral cilia on *Mytilus* gill is due to 5 HT-triggered augmentation of cellular cAMP levels, leading to cAMP-dependent protein kinase-mediated phosphorylation of axonemal dynein light chains (Stephens and Prior, 1990). Quiescence of lateral cilia is thus believed to reflect lowered cAMP concentration and resultant dephosphorylation of dynein polypeptides. Phosphorylation of axonemal dynein polypeptides has also been reported in other systems (Hamasaki and Satir, 1989; Chilcote and Johnson, 1990; Dey and Brokaw 1991; Stephens and Prior, 1991).

We found that membrane-permeant cAMP analogs stimulate the beating of *Ciona* stigmatal cilia held in a long-lasting inactive state (termed quiescence). Moreover, reactivated beating of stigmatal ciliary models is improved by cAMP and IBMX, or the addition of the catalytic subunit of protein kinase to RS. Reactivation of *Mytilus* lateral ciliary models is also improved by the presence of the catalytic subunit, which can override Ca arrest (Stommel, 1984; Stommel and Stephens, 1985; Stephens and Stommel, 1989). These findings suggest that the quiescence (long-lasting inactivation) of *Ciona* cilia is physiologically similar to the quiescence of *Mytilus* lateral cilia; *i.e.*, that increased cAMP levels may also be responsible for maintaining the activity of *Ciona* stigmatal cilia via cAMP-dependent phosphorylation of regulatory axonemal polypeptides.

Although their underlying biochemical mechanisms seem to be similar, the postures of quiescent *Mytilus* lateral cilia and quiescent *Ciona* sigmatal cilia are quite different. Lateral cilia rest at the end of the recovery stroke, whereas stigmatal cilia stand upright, midway between the effective and recovery strokes.

Ciona cilia thus remain upright during both the transient inactive state and the quiescent or long-lasting inactive state. However, quiescence differs from inactivation, not only by its longer duration, but also by the apparent absence of a preceding arrest.

cAMP and Ca act antagonistically on *Mytilus* lateral cilia, as well as on several other ciliary and flagellar systems (see Brokaw, 1987; Stephens and Stommel, 1989; Bonini *et al.*, 1991). Moreover, cAMP or cAMP-dependent protein kinase can override the Ca effect and activate beating of Ca-arrested cilia (Murakami and Takahashi, 1975; Murakami, 1983; Stommel and Stephens, 1985). Our finding, that adding Ca and cAMP to RS causes rapid vibration or twitching of stigmatal cilia in the straight inactive position, indicates a similar antagonism between cAMP-mediated activation of beating and Ca-induced inactivation (rather than arrest) of cilia.

In conclusion, we have developed an *in vitro* preparation of *Ciona* branchial basket cilia which allows investigation for the first time of the ionic and molecular control of ciliary motility in tunicates. Further studies using improved models should provide more detailed and quantitative information for comparison to other systems.

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